PROTOCOLS

Production of a heterologous protein in bioreactor cultures of fully differentiated moss plants

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Abstract

A wild-type strain of the moss *Physcomitrella patens* was genetically transformed with the bacterial *gus*-gene under control of 35S cauliflower mosaic virus promoter at rates of about 2.4×10^{-3} for transient and 1×10^{-5} for stable transformants. Five of the latter had integrated 1–5 plasmid molecules, mainly as concatemers. Transgenic plants expressing the *gus*-gene were used to establish a mass culture in a foil-bioreactor. Biomass doubled every 39 to 42.5 hours in terms of total protein and every 55 hours in terms of total chlorophyll. Activity of β-glucuronidase increased overproportionally until harvest of the culture.

Introduction

The production of proteins in foreign organisms is of increasing importance; most being produced in prokaryotes. However, eukaryotic proteins may not be modified correctly in prokaryotes and therefore, increasing numbers of eukaryotic cell systems like yeast or mammalian cell cultures are used for this purpose (Harford, 1988; Biocca *et al.*, 1990). Cell cultures of higher plants have been used for the production of secondary metabolites (Berlin *et al.*, 1994). However, *in vitro* cultures of plant cells frequently show somaclonal variations, leading to loss of product formation.

In contrast, differentiated plants of the moss *Physcomitrella patens* can be grown in plain inorganic liquid media (Reski and Abel, 1985). Here we report on the construction of transgenic moss plants expressing the bacterial *gus*-gene, establishment of a bioreactor culture with fully differentiated transgenic plants, and the production of a heterologous protein in these cultures.

Materials and methods

Plant material. The wild-type strain of *Physcomitrella patens* (Hedw.) B.S.G. has been characterized previously (Reski *et al.*, 1994). Plants were grown under controlled conditions in a plain inorganic medium devoid of any phytohormones or vitamins (Reski and Abel, 1985). 1 g of 6–10 days old plant material was digested by agitated incubation for 15 hours in the dark in 0.5% Driselase (Sigma) / 0.5M mannitol. This suspension was passed through sieves (Wilson) of 100 μm and 45 μm pore size and centrifuged for 5 minutes at 70xg. Pellets were solubilized in 10 ml of 20% sucrose in

CPW salts (Banks and Evans, 1976), topped with 0.5 ml W5 solution (Menczel *et al.*, 1981) and recentrifuged for 10 minutes at 150xg. Protoplasts from the interface were washed twice by centrifugation in W5.

Protoplast transformation.   Plasmid p35SGUS (Schmülling *et al.*, 1989) was isolated and purified via Qiagen Tip-500 columns (Diagen, Hilden) and was resuspended in 0.1 M $Ca(NO_3)_2$ at a concentration of 0.5 µg/µl. 100 µl of DNA-solution was transferred to a sterile glass tube.   Freshly isolated protoplasts were counted and resuspended at 1.2×10^6/ml in 3M-medium (Schäfer *et al.*, 1991). 250 µl of protoplast-suspension was added to the DNA and mixed gently, followed by the addition of 350 µl PEG-solution (40% PEG_{4000} in 3M-medium, pH 6.0).   The transformation mix was incubated at room temperature for 30 minutes with occasional gentle mixing. Subsequently, the solution was diluted by adding 1 ml, 2 ml, 3 ml and 4 ml 3M-medium every 5 minutes. Protoplasts were pelleted (5 min., 70xg), resuspended in regeneration medium (Rother *et al.*, 1994), transferred to 3 cm petri dishes and were incubated for 24 h in dim light (4.9 $\mu mols^{-1}m^{-2}$). After that, cultures were transferred to normal growth conditions (46.8 $\mu mols^{-1}m^{-2}$; light-dark regime of 16/8 h; $25 \pm 1°C$) for 5–6 days. Outgrown protoplasts were transferred to a cellophane disc on solidified Knop-medium (Reski and Abel, 1985) supplemented with 50 mg/L G418. Antibiotic-resistant plants could be isolated 24 days after transformation. Plant DNA was isolated according to Reski *et al.* (1991), transferred onto nylon membranes (Hybond N^+, Amersham) according to standard protocols (Sambrook *et al.*, 1989) and was hybridized to DNA-probes labeled with the DIG (digoxigenin) random-primed labeling kit (Boehringer, Mannheim, Germany).

Bioreactor culture.   Plants were cultivated for 14 days in 300 ml liquid Knop-medium and were disrupted to filaments of about 10 cells with an ultra turrax prior to inoculation.   The bioreactor culture was performed in a 2L foil-bioreactor (Bioengineering, Bern, Switzerland) with 1.8L Knop-medium under controlled conditions (constant pH 5.8, 97.5 $\mu mols^{-1}m^{-2}$, a light-dark regime of 16/8 h, 25°C). Air was supplied through stainless steel tubes and plants were moved by stirring at 500 rpm. Every second day protonemata were disrupted by stirring for 2 minutes at 2,000 rpm. Every second or third day 30 ml-samples were collected and the removed medium was replaced by freshly prepared medium. Quantification of β-glucuronidase activity followed the protocol of Jefferson (1987). Additionally, extracts were purified by a Sephadex G25-column (Pharmacia) to remove polyphenols. 50 µl of the GUS-extract was incubated with 800 µl 0.1N NaOH and 200 µl Bio-Rad reagent (Bio-Rad, München, Germany) for 5 minutes at room temperature before proteins were quanitified at 578 nm. Pigments were extracted with 80% acetone (2 ml per 200 mg), supplemented with 50 mg $MgCO_3$. The homogenate was incubated for 5 minutes on ice, centrifuged (5 minutes, 600xg, 4°C) and the pellet was reextracted for further 5 minutes with 80% acetone. Liquid phases were pooled and absorbency was determined at 664 nm, 647 nm and 750 nm, respectively. From that, total chlorophyll content was calculated according to Ziegler and Egle (1965).

Results

Transformation of Physcomitrella patens. *P. patens* was genetically transformed via the PEG-mediated direct DNA transfer with a 11.5 kb plasmid, carrying the *E. coli gus*-gene under control of the cauliflower mosaic virus (CaMV) 35S-promoter as well as the *npt*II-gene under control of the *Agrobacterium tumefaciens* nopalin synthase promoter. Transformants were obtained at frequencies of 2.4×10^{-3} according to growth on selective media. These plants were grown for 14 days on non-selective media and than retransferred to selection pressure. In four independent experiments twelve plants survived this treatment. As judged by Southern-blot analyses all survivors had integrated the foreign plasmid. As 3×10^5 protoplasts had been treated with the DNA-solution per experiment, stable transformants were obtained at frequencies of 1×10^{-5}. These transgenics did not show any phenotypic difference to untransformed wild-type plants, proved to be fertile and inherited the transgenes as haploid, self fertile plants to 100%. According to Southerns from five transformants, one to five copies of the plasmid was integrated per genome, mainly as concatemers (Fig. 1).
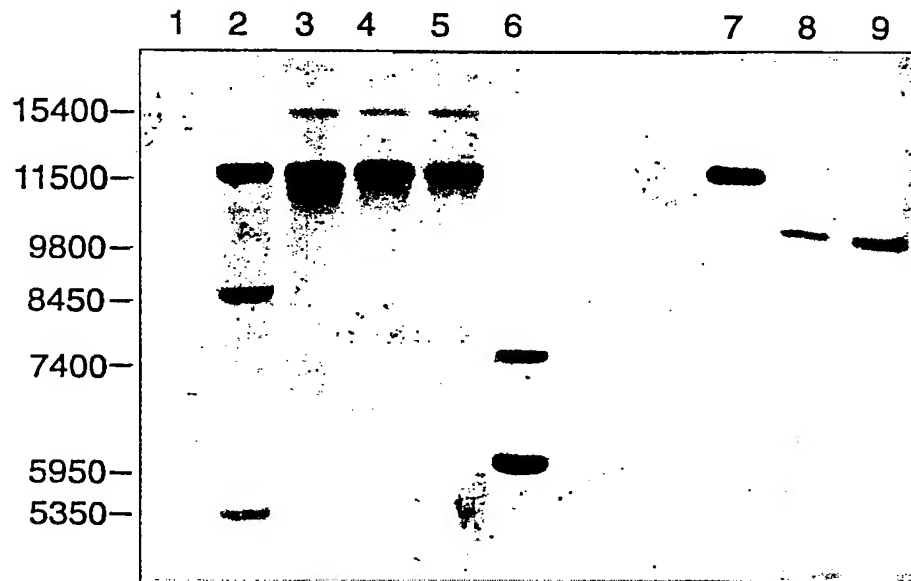

Figure 1. Southern analysis of five different transformants and one untransformed control plant. Genomic DNA was digested with *Hin* dIII. A digoxigenin-labeled *Sal* I fragment of p35SGUS was used as a probe. 1 = control, 2–6 = transgenics; 7 = p35SGUS digested with *Hin* dIII; 8, 9 = p35SGUS undigested, corresponding to 1 and 3 plasmid copies per genome, respectively. Sizes are given in base pairs (bp).
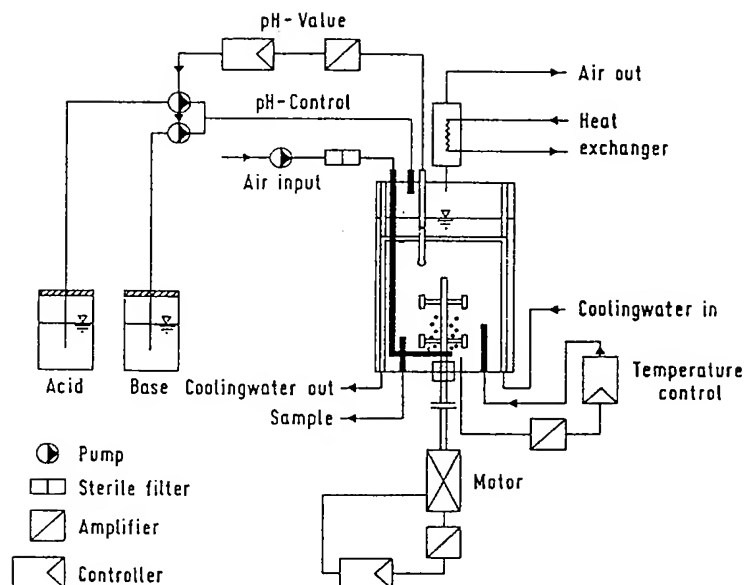
Figure 2. Schematic drawing of a 2L foil-bioreactor (Bioengineering, Bern, Switzerland), used in this study to cultivate fully differentiated moss plants.
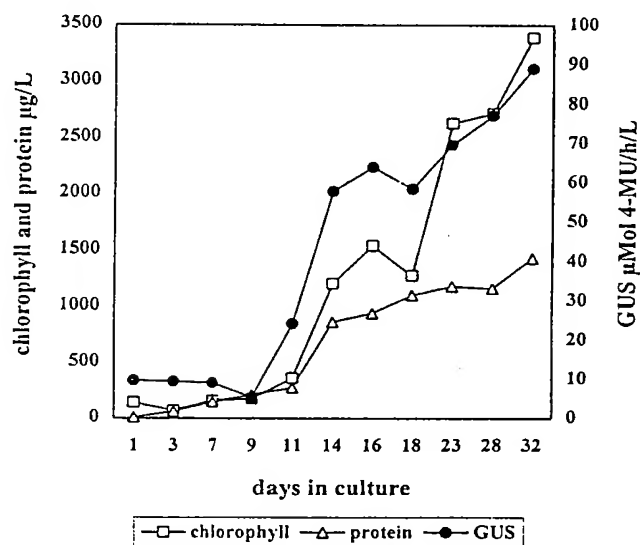

Figure 3. Growth characteristics of transgenic moss plants in the bioreactor culture, in terms of total chlorophyll and total protein content, as well as β-glucuronidase production scored during 32 days from 30-ml samples.

Plant growth and production of β-glucuronidase. One transgenic (Fig. 1, lane 2) was axenically grown in a 2L foil-bioreactor under controlled conditions (Fig. 2). Seven days after inoculation protonemata developed many new side branches with short, stunted cells, which grew out to form protonema balls. This development was enhanced by periodically high speed stirring. Twenty days post inoculation a high production of

leafy shoots was observed. Foaming of the medium never occurred. Biomass increase was monitored in terms of total chlorophyll and total protein content during a 32 day-period. Total protein content doubled every 39 to 42.5 hours, while chlorophyll doubled every 55 hours. The activity of β-glucuronidase increased tenfold from day 9 until harvest of the culture (Fig. 3). Although absolute values varied in repeated experiments, the shape of the curves were reproducible.

Discussion

In plants, heterologous proteins with potential economic value were either produced in-vitro in undifferentiated cells or in greenhouse grown plants (Hiatt *et al.*, 1989; Düring *et al.*, 1990; Krebbers and Vandekerckhove, 1990). However, callus cells are often genetically unstable and production of proteins in greenhouse plants may not meet pharmaceutical quality standards. Potentially combining the advantages of both systems, fully differentiated moss plants can be grown axenically in a non-expensive, plain liquid medium of inorganic salts (Reski and Abel, 1985). So far, mass cultures were performed in batch-techniques with moss plants being circulated by airlift (Boyd *et al.* 1988; Marienfeld *et al.*, 1989; Rudolph and Rasmussen, 1992). We report that intact moss plants can be cultivated in a bioreactor agitated by a stirrer. According to growth characteristics they resemble batch cell cultures of higher plants: cultures start with a short lag-phase, followed by a nearly linear growth. However, in the present experiments, plants were harvested before reaching the stationary phase.

So far, there have been no reports on the production of a heterologous protein in moss mass cultures. The increase of β-glucuronidase activity paralleled the increase in biomass as long as plants were in the protonemal phase. Upon gametophore production, however, enzyme activity increased overproportionally, most likely due to a developmental control of the 35S CaMV promoter, as has been reported for transgenic higher plants (Benfey and Chua, 1990). As production of leafy shoots in mosses is under hormonal control and, furthermore, cultures were harvested before reaching the stationary phase, we currently optimize protocols for the production of heterologous proteins in moss plants.

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